STUDYING THE ARSENIC ABSORPTION BY KERATIN PROTEIN EXTRACTED FROM HUMAN HAIR

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF

Bachelor of Technology
In
Biomedical Engineering
By

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NATIONAL INSTITUTE OF TECHNOLOGY
ROURKELA

CERTIFICATE

This is to certify that the thesis entitled, **"*STUDYING THE ARSENIC ABSORPTION BY KERATIN PROTEIN EXTRACTED FROM HUMAN HAIR*"** submitted by **Ms. Swati Paswan** in partial fulfilment of the requirements for the award of the Bachelor of Technology in Biotechnology and Medical Engineering with specialization in "Biomedical" at National Institute of Technology, Rourkela is an authentic work carried out by her under my supervision and guidance.

To the best of my knowledge, the matter embodied in this thesis has not been submitted to any other University/ Institute for the award of any other Degree or Diploma.

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ABSTRACT

Elevated levels of the element arsenic in the human body cause Arsenic poisoning. A strong correlation exists between the intake of Arsenic and harmful health effects. While Arsenic exposure can occur from air, food and water, major Arsenic poisonings have been caused by water and this, in most cases is the predominant exposure route. **E**levated arsenic concentrations occur naturally in deeper levels of groundwater. In consequence, removing Arsenic from water has become extremely necessary. In water it is present majorly present in trivalent and pentavalent form. The trivalent form stays as a neutral species in water, making it extremely difficult to eliminate. Before subjecting it to any other removal techniques, it is generally oxidised to pentavalent state.

Arsenic gets deposited on certain sites of the human body, such as hair, nails, skin etc. This indicates the Arsenic absorptive ability of human proteins. This remarkable property of proteins propelled us to study and analyse more.

Human hair which is normally a waste product has been used to study the Arsenic absorptive property of the keratin protein and its efficacy as a water purifier. Here we extract proteins from human hair, to create human hair matrix for the study of trivalent arsenic absorption by the de-proteinized hair. A solution of arsenic trioxide is used as test solution to ensure the presence of arsenic in trivalent form. Shindai method is used for protein extraction. The extracted protein is characterised by using several techniques such as Electrophoresis, X-Ray Diffraction, Scanning Electron Microscopy, Differential Scanning calorimetry, Fourier transform Infrared spectroscopy and Energy-dispersive X-ray spectroscopy. The increased concentration quantification and thus the absorption of Arsenic on the hair is tested by the Arsen 10 Test.

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CHAPTER 1

INTRODUCTION

1.1 Arsenic

Arsenic is a chemical element with the symbol **As**, whose atomic number is 33 and it has relative atomic mass 74.92. Arsenic can exist in the form of various allotropes and is mostly considered monoisotopic. Arsenic and many of its compounds are seen to be potent poisons. These poisons contaminate many water supplies. 10 ppb for drinking water and 5 ppb for bottled water is the maximum allowed concentration of As [1]. Elemental arsenic and its compounds are classified as toxic and dangerous for the environment.

1.2 Arsenic Toxicity & Poisoning

The oxides of arsenic are the most common hazard since arsenite (Trivalent- AsO_3^{3-}, AsO_2^-) and arsenate (Pentavalent- AsO_4^{3-}) salts are extremely poisonous. Arsenic poisoning can be caused due to human actions such as mining, ore smelting, etc. Water wells drilled into aquifers having high concentrations of arsenic are the most serious problems. Groundwater As affects many millions of people. Food is the less toxic organic arsenic source, with the predominant source being seafood. "Inorganic arsenic" (arsenate and arsenite salts) are 500 times more harmful than organic arsenic exposure. By the method of allosteric inhibition, Arsenic interferes with the cellular longevity of metabolic enzyme pyruvate dehydrogenase (PDH) complex. This enzyme catalyzes the oxidation of pyruvate to acetyl-CoA byNAD+. The energy system of the cell is disrupted causing cellular apoptosis. Inorganic Arsenic trioxide in ground water particularly affects voltage-gated potassium channels, which disrupts the cellular electrolytic function resulting in neurological disturbances, cardiovascular episodes such as prolonged Q-T interval, anemia, central nervous system dysfunction, neutropenia, high blood pressure, leukemia, and death.

1.3 Groundwater Arsenic Occurrence

Arsenic is a minor constituent of some common minerals, and the concentration of dissolved arsenic is more than 1 µg/L are common in groundwater. Much greater arsenic concentrations can be found in some aquifers and under certain conditions and concentrations above 1Oµg/L are quite common. Environmental Protection Agency standard, accepting only 10 parts per billion of As concentration in drinking water was adopted in 2001[2]. Asia, including locations in Thailand, Taiwan, and Mainland China, also South America, Argentina and Chile and many locations in United States are affected.

1.4 Treatment Methods for arsenic containing water

Ion Exchange Method

Researchers have shown that this method can reduce arsenic (V) concentration to a value below 2µg/L. It is effective even when the sulfate concentration is as high as 200 mg/L. The research also suggested that, if some salt was added to the solution to provide sufficient chloride levels, the brine regeneration solution could be used again and again with no degradation in arsenic removal [3]. Brine recycle reduces the cost of operation as well as the amount of waste for disposal.

Coagulation and Direct Filtration

Arsenic (V) can be consistently removed to a concentration level as low as 2 µg/L by the method of iron addition (coagulation) and subsequently by direct filtration (microfiltration system) [4] .Critical operating parameters are iron dose, pH, detention time and mixing energy.

Conventional Iron/ Manganese (Fe/Mn) Removal Processes

Iron coagulation/ filtration, together with addition of iron with direct filtration means is an efficient method for arsenic (V) removal. By making the use of conventional Fe/Mn removal processes, source waters can be treated for arsenic removal. The source water contains naturally occurring iron and/or manganese and arsenic. These procedures can notably reduce the arsenic by eradicating the iron and manganese from the source water. This removal technique is based upon the same mechanism that takes place with the iron addition methods.

Reverse Osmosis

It is a filtration procedure that makes the use of membrane-technology. Pressure is applied to the solution when it is on one of the sides of a selective membrane. It helps in the removal of various types of larger molecules and ions from the solution. The effect is that the solute is reserved on the pressurized membrane side and the pure solvent is let to flow to the other side. For attaining selectivity, large molecules or ions should not be allowed to pass through the membrane through the pores (holes). Only the smaller components of the solution should be allowed to pass without restraint.

1.5 Demerits of the treatment techniques

Coagulation/Filtration and Lime Softening:

- Not suitable for most small scale systems-- need for trained operators, high cost, and variability in performance.
- Sludge disposal may be a problem

Activated Alumina:

- In case of small systems the process becomes too intricate and dangerous because of the intensive requirement of proper chemical handling.
- In the long term, activated Alumina may not be efficient. It loses considerable adsorptive capability with each cycle.
- Brine disposal may be a problem.

Ion Exchange:

- Brine disposal may be a problem. Brine recycling might reduce the impact.
- Run length is affected by Sulphate level.

Reverse Osmosis/ Nanofiltration:

- Extensive corrosion control is required for low-level option.
- Water rejection (about 20-25 % influent) may be an issue in water-scarce regions

CHAPTER 2

Literature Review

2.1 Human Hair Structure

Hair, a biological polymer has as much as over 88 % of its dry weight made up of Keratin Protein. Keratins contain cystine, forming two groups of proteins - Intermediate filament proteins and Intermediate filament associated proteins (Powell et al. 1991) that are almost equally abundant in most hairs. Human hair comprises of fibrous a-keratin proteins. The three morphological regions are - the cuticle, cortex and the medulla. Hair fiber result from compact groups of cells within the fiber follicle and are not continuous. [5, 6]

Cuticle

It is a protective layer which covers the core of the fibres. It exhibits a scaled structure and is generated by a single layer of cells. It is majorly composed of b-keratins. It possesses seven to ten layers superimposed onto each other. The cuticle edges point toward the tip of the fibre [7]. The three basic scale structures of the cuticle are — the coronal or crown-like structure, the spinous or petal-like, and the imbricate or flattened structure. Epicuticle is a thin membrane coating on the cuticle scale cells. Exocuticle, containing majority of the cystine in the scale is covered by Epicuticle [8]. Interface of the cortex locates the Endocuticle. Endocuticle is composed of the remaining cell organelles and has low cystine residue content [9]. The thickness of a cuticle cell is approximately 0.3 to 0.5 µm and length is approximately 5 to 10 µm.

Cortex

It constitutes the most voluminous part of the hair fiber. Tightly surrounded by the cuticle, the Cortex is formed by spindle-shaped (fusiform) cells. Entities such as pigment granules, cortical fusi and large oval/round structures known as ovoid bodies are present in the cortex. The corticle cells make up the cortex. Long filaments known as the Macrofibrils comprise the corticle cells. The intermediate filaments, known as the Microfibrils form the Macrofibrils [10, 11].

In The cortex, the hair fiber can be divided into two main groups of protein:

(1) Low Sulphur, alpha keratins – Molecular Weight – 40 to 60 kDa.

(2) High Sulphur, matrix proteins - Molecular Weight – 10 to 25 kDa.

Hence, overall the hair fiber comprises of 50 to 60 % of alpha keratins and 20 to 30% of matrix proteins [12]. Keratin intermediate filaments (KIFs) are formed by the assembling of alpha keratins. KIFs are micro-fibrous structure that lends toughness to the hair fiber. The matrix proteins are also known as keratin associated proteins (KAPs). The cortical superstructure is held together by KAPs functioning primarily as a disulfide crosslinker [13].

Medulla

These are small membrane-bound cavities within a cell which may exist along the axis of alpha Keratin fibre. Such cells constitute a very small percentage of the hair mass. They are also believed to contribute negligibly to the mechanical properties of the fibre. Air fills the Medulla [6, 9]. Under transmitted light it appears as a black or opaque structure, or as a white structure under reflected light. In human hair, Medulla generally exhibits amorphous characteristics.

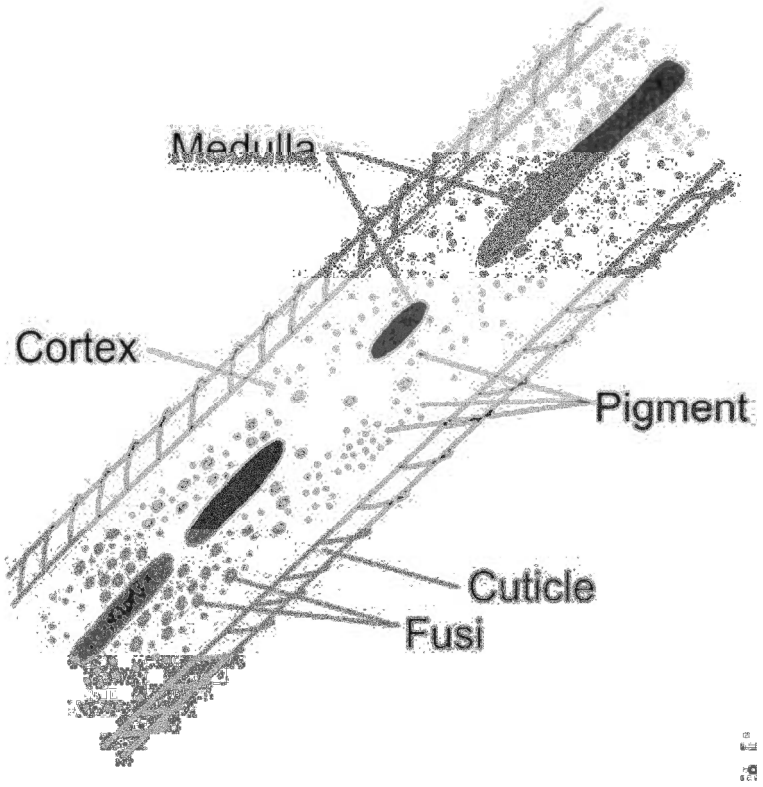

Figure 1: Cross section of a hair shaft [14].

2.2 Chemical Bonds in Hair

The proteins called Keratins comprising of polypeptide chains are hard fibrous type. The alpha helix is the term given to the polypeptide chain forming the keratin protein found in human hair is known as alpha helix. It is a coiled structure. The coils are formed by the amino acids linked together. Approximately 3.6 amino acids are present in one turn of the helix (coil). Amino acids are connected together by the virtue of peptide bonds. The peptide bond is situated between the carbon atom of one amino acid to bond with the nitrogen atom of the next [15].

The Alpha Helix Coil

Organisation of a single here comprises of "protofibril", which is formed when three "alpha helices" are twisted together. An eleven-stranded cable recognized as "microfibril", is formed by nine protofibrils then bundled in a circle around two or more. Amorphous protein matrix with high sulfur content embeds the microfibrils. This arrangement leads to the formation of a structure called "macrofibril". The cortex layers of the hair fiber are formed by the grouping of these macrofibers. The cuticular layers of the hair packed dead cells surround these structures. The medullary canal lies in the center of these structures.

Bonding in Keratin Protein

The original make-up of the hair stays by the bonding found in the cortex of the hair. Each and every alpha helix comprises of these bonds.

The Hydrogen Bond

This bond is positioned between the coils of the alpha helix and is accountable for its elasticity. These bonds are electrolytically controlled and are the bonds that are most readily broken down and again are the bonds that are the most readily reformed. These bonds are responsible for approximately 50% of the hair's elasticity and 35% of the strength of the hair.

The Salt Bond

It is an ionic bond formed by the electron transport from the side chain of amino group, which is basic (an amino acid with an 00C- group) to an amino acid side chain (acidic). This occurs at a point which is paralleled to the axis line of rotation of the helix of hair.

Generally 35% of the strength of the hair and 50% of the hair's elasticity comes from the salt bond.

The Cystine Bond

These are also known as the disulfide bond. These are formed by cross-linking among cystine residues (amino acids) of the polypeptide chain. Cystine bond is always formed perpendicular to the axis of the hair and between the polypeptide chains. Hair gets its toughness or abrasion resistance on account of this position in the hair. These cross-linkings in the hair fiber are frequent.

The Sugar Bond

The sugar bond is particularly formed between an acidic amino group the side chain of an amino acid, having OH group. This bond is perpendicular to the axis of the hair too. By the virtue of its position, it confers the hair its toughness but only little strength (5%). As a by-product of this bonding some moisture is contributed to the hair.

2.3 Keratin

"Keratin" is a term that was originally referred to a category of insoluble proteins. Keratins make up the bulk of cytoplasmic epithelia and epidermal appendageal structures, which are the hair, wool, nails, horns and hooves. Keratin proteins get associated as Intermediate Filaments. Based on their structure, regulation and function, mammalian Keratin can be classified into two distinct groups as- Hard keratin and Soft Keratin.

Hard keratin

These contribute in forming the tough structure of epidermal appendages. Hard keratins are made up of ordered arrays of intermediate filaments. These filaments are embedded in a cystine rich matrix.

Soft Keratin

These give the epithelial cells the property of mechanical resilience. The soft keratins are made up of bundles of cytoplasmic intermediate filaments, which are loosely packed.[16, 17, 18]. The epithelial and hair keratins have structural subunits. Both have two chains which differ in molecular weight and composition. The two subunits are designated as-

(1) Type I – acidic.

(2) Type II- neutral to basic.

Each one of these types comprises of a central alpha-helical domain and a non-helical end terminal domains. The interaction of type I and type II keratin chains form heterodimers. 10-nm intermediate filaments are formed by the polymerisation of the heterodimers. The amount of cystine residue content is higher in non- helical domains of hair keratins. Thus, due to the intermolecular disulfide bond formation, the hair gets tougher and more durable structures [18, 19, 20].

Hair Keratins

In total, there are 17 human hair keratin genes (11 type I; 6 type II) [21] and more than 85 KAP genes [21] that potentially contribute to the hair structure in humans.

2.4 Isolation of Keratin Proteins

Isolation of keratin from Cultured Cells with Fibroblastic Morphology

Intermediate filaments (IF) which are conventionally expressed as fibroblasts were isolated from a variety of cultured cells. Intermediate filaments are composed of proteins, which have the molecular weight of 54,000 or 55,000. The percentage of the protein established, in native Intermediate Filament arrangements from these cells and comprising of three to four

polypeptides (molecular weights 60,000-70,000), is less than 15 %. BHK-21 and mouse 3T3 cells were used to isolate these proteins. Some immunological and biochemical properties of these proteins have been investigated. It has been found that, these proteins are capable of constituting paracrystals. When stained (negatively) with uranyl acetate, the paracrystals show light or dark banding patterns. The dark bands are of the 2-nm-diam filaments, which are longitudinally aligned. . The centre - centre spacing between either light or dark bands is found to be 37-40 nm. Secondary structures of IF polypeptides show similar dimensions. Thus the values are consistent. The direct inference drawn from the analysis is that the lateral alignment of alpha helically coiled domains is represented by the dark bands. The data acquired from secondary structure, immunoblotting and amino acid composition indicate these paracrystal polypeptides are similar to keratin. Thus, it was concluded that polypeptides with immunological and biochemical properties of epidermal keratin are there in cells , that are usually considered to be fibroblasts[22] .

Differential Extraction of Keratin Subunits and Filaments from Normal Human Epidermis

In vivo keratin interactions were investigated and studied. Sequential extraction of water-insoluble proteins from epidermis was executed. The concentration of urea was increased in a step-wise manner as 2 M, 4 M, 6 M and 9.5 M. 1-2 Dimensional gel electrophoresis, immunoblot examination by means of monoclonal anti-keratin antibodies, and EM were done for examining each extract. The layers of human epidermis contain keratins K1, K2, K5, K10/ll, K14, and K15. During the course of epidermal differentiation these keratins were sequentially expressed. Primarily keratin filament subunits were revealed by staining (negative) of the 2-M urea extract. In the 4-urea and 6-M urea extracts, large amount of intermediate-sized filaments were observed. The differentiation-specific keratins KI, K2 and

K10/ll were less soluble than keratins K5, K14, and K15. This finding implies that native keratin filaments of dissimilar polypeptide composition have different properties, despite their similar morphology [23].

Chicken feather keratin: **extraction, characterization and films preparation**

The aim of the study was to extract and to establish the amino acids profile, average molecular weight and the thermal stability of chicken feather keratin .The basic properties of the films obtained were also analysed. The extraction procedure included materials such as an aqueous solution of urea, 2-mercaptoethanol and surfactant of pH 9.0. The process helped in the extraction of 94% of the keratin that was present in the dry feather. The molecular weight of keratin was determined by electrophoresis method. The result obtained was ranging from 10 to 14,000 g mol^{-1}. The degradation temperatures of keratin were not changed by the extraction procedure. This result was shown by Thermogravimetric analyses. The films of feather keratin were compared with the films of wool keratin mentioned by the literature. It was observed that the films of feather keratin exhibited lower tensile strength than the films of wool keratin. [24]

CHAPTER 3

Materials and Methods

A: Shindai Solution Preparation and Arsen 10 Testing

MATERIALS

Chemicals Required

Ethanol

Methanol

Chloroform

Urea

2-Mercaptoetanol

Glutaraldehyde

Thiourea

HCl

Arsenic trioxide

Distilled water

Tris- base

Glassware Required

Beakers

Flat bottom flasks

Funnels

Spatulas

Pipettes and pipette tips

Filter papers

Labware Required

Weighing balance

Incubator

Desiccators

Centrifuge machine

METHOD

Human hair are de-proteinized by **Shindai method** of protein extraction. The extracted hair sample is used as an absorption material.

Hair protein extraction :

Delipidizing the hair

1. Hair is cut into small pieces of approximate length 2 mm.
2. Hair is treated with ethanol for 30 seconds in order to clean.
3. Hair is delipidized using 2:1v/v solution of chloroform and methanol. The time of treatment is 24 hours.
4. The solution is drained and hairs are dried.

Protein extraction

5. Shindai solution is prepared using following constituents:

Urea	5M
Thiourea	2.6M
Mercaptoethanol	5%
Tris-base	25mM

6. Two flat-bottom flasks are filled with 50 ml solution prepared above. Their pH is adjusted to 8.5.
7. Add 10% SDS to one of the flasks (modification). The other flask acts as control.
8. Add 4 gm of delipidized hair to each flask. Incubate at 50^0 C for 72 hours.
9. Filter by means of filter paper. The filtrate contains extracted protein.
10. The hair sample obtained on the filter paper is dried and matrix is prepared from it by using Glutaraldehyde.

11. A tablet from this hair matrix is formed.

12. The tablet is used as a filter for passing Arsenic solution and Arsen 10 test was run to determine Arsenic absorption.

13. The filtrate obtained in Step 9 is centrifuged at 1500g, for 15 minutes and at 4^0 C.

14. The samples were then dialysed for 5-6 hours against:

Tris/HCl (pH 7.5)	5mM
DTT	1m

15. Characterization techniques such as XRD, SEM, FTIR, EDX and DSC of the protein obtained were conducted.

B : Scanning electron Microscopy

Scanning Electron microscope is a type of electron microscope. The sample is scanned with a beam of electrons. Signals are produced when the electrons interact with the atoms of the sample. This interaction produces signals that contain information about the sample's composition, surface topography, electrical conductivity, crystalline structure and orientation of sample materials. Data is collected over a certain area of the exterior of the sample, and a 2-dimensional image, displaying the properties is generated.

Sample Preparation

At the surface specimens should be electrically conductive and electrically grounded. This avoids the gathering of electrostatic charge at the surface. Nonconductive samples have a tendency to charge when scanned by the electron beam. They are consequently coated with a thin coating of a conducting material, which is deposited on the sample either by high-vacuum evaporation or low-vacuum sputter coating.

Measurement Conditions

Delipidized Hair

Tilt (deg) : 0.0

Elevation (deg) : 35.0

Azimuth (deg) : 0.0

Magnification : 100 X

Accelerating voltage (kV) : 20.00

Process time : 5

Deproteinized Hair

Tilt (deg) : 0.0

Elevation (deg) : 35.0

Azimuth (deg) : 0.0

Magnification : 150 X

Accelerating voltage (kV) : 20.00

Process time : 5

Extracted Protein

Tilt (deg) : 0.0

Elevation (deg) : 35.0

Azimuth (deg) : 0.0

Magnification : 250 X

Accelerating voltage (kV) : 20.00

Process time : 5

C: Fourier Transform Infrared Spectroscopy

It is a method which is used to attain an infrared spectrum of absorption, emission, and photoconductivity. Fourier transform is required for the conversion of the untreated data into the definite spectrum.

Sample Preparation

The sample can be prepared by either placing 2-5 mg of compound on the IR plate and addin a drop of solvent over it, or by dissolving the compound in a small test tube and transferring it onto the IR plates by a pipette.

First the KBr plates are cleaned to prevent contamination of future samples. The windows are wiped with a tissue and then washed several times with the solvent and later with ethanol. The windows are then polished using the polishing kit in the lab.

D : X- ray Diffraction

It is a non-destructive analytical technique which reveals the information about the crystal structure, chemical composition, and physical properties of materials and thin films. These techniques are based on observing the scattered intensity of an X-ray beam hitting a sample as a function of incident and scattered angle, polarization, and wavelength or energy.

Measurement Conditions:

Table 1 -Measurement Conditions of XRD

Sample Identification	S. PASWAN BTECH PROJ
Comment	Exported from X'Pert software
Measurement Date / Time	4/26/2012 14:10

	PHILIPS-binary (scan) (.RD)
Raw Data Origin	Gonio
Scan Axis	5.0100
Start Position [°2Th.]	49.9900
End Position [°2Th.]	0.0200
Step Size [°2Th.]	0.4000
Scan Step Time [s]	CONTINUOUS
Scan Type	0.0000
Offset [°2Th.]	Fixed
Divergence Slit Type	1.0000
Divergence Slit Size [°]	10.00
Irradiated Length [mm]	10.00
Specimen Length [mm]	2.0000
Receiving Slit Size [mm]	0.00
Measurement Temperature [°C]	Cu
Anode Material	30 kV, 20 mA
Generator Settings	XPert MPD
Diffractometer Type	1
Diffractometer Number	200.00
Goniometer Radius [mm]	91.00
Dist. Focus-Diverg. Slit [mm]	No
Incident Beam Monochromator	No
Spinning	

E :Energy-dispersive X-ray spectroscopy

EDS or EDX is an methodical technique which is used for the analysis of the element or characterization of the sample. It requires

Interaction between a X- ray excitation source and a sample. Since each element has its unique atomic structure which allows unique set of peaks on its X-ray spectrum. A high energy beam of charged particles is focused on the sample to studied, so that simulation of characteristic emission of X-ray can occur. EDX allows the measurement of the elemental composition.

F: Differential Scanning Calorimetry

DSC is a thermo-analytical technique in which the difference in the amount of heat needed to increase the temperature of a reference and a sample is measured as a function of temperature. Both the reference and sample are kept at approximately the same temperature. The temperature of the sample holder increases linearly with time.

Sample Preparation

Here the sample weight plays an important role. The optimum selection of weight of the sample so that it is a representative of the total sample is significant. The change in heat flow in the sample due to transition of interest should be around 0.1 -10 mW.

Measurement Conditions

Temperature Range : 25 ºC to 400 ºC

Rate : 5 ºC / minute

G: QUANTOFIX Arsenic 10 (As Testing Kit)

Quantofix Arsen 10 testing kit was used to compare the concentration of arsenic in the solution. This is a colorimetric analysis kit wherein the sample is taken in testing bottle and reagents are mixed. The test strip provided has the test area which is exposed to vapours arising from the occurring reaction. If the sample contains arsenic the test area changes coloration and it can be compared to the reference color chart available with the kit. The range of arsenic concentration can easily be known and also the concentration of arsenic in two or more solutions can be compared using this.

Contents

1 Aluminium container with 100 test sticks

1Bottles Arsenic-1

2 Bottles Arsenic -2

1 Reaction Vessel

1 Syringe 10 ml

1 needle

Hazards Information

Each test field on the test stick contains mercury (II) bromide. Toxic by contact with skin.

Procedure

1. Use syringe to add 2 x 10 ml sample solution into the reaction vessel.
2. Add 1 measuring spoon reagent Arsenic-1 and swirl reaction vessel gently.
3. Add 1 measuring spoon reagent Arsenic-2.

4. Insert test strip with test field 2 cm into reaction vessel and clamp it with lid.
5. For pressure balance, prick the needle through the lid and leave it there.
6. During 30 minute reaction time, gently swirl the reaction vessels. The test field should not get in contact with the sample.
7. After 30 minutes remove test strip from reaction vessel, dip it for 2 seconds into distilled water. Shake off excess liquid and compare test field with colour solids.

Measurement Conditions

Flow rate - 1 ml/min

Quantity of Hair Protein - 1 g

Volume – 25 ml

CHAPTER 4

Results and Discussions

4.1 Scanning Electron Microscopy

Scanning Electron Microscopy of samples – Delipidized hair, Deproteinized hair and Extracted protein sample was done. SEM helped in knowing the topography of all the three samples. It also helped in noting the changes that occurred in the hair during the whole procedure of sample preparation and protein extraction using Shindai Method.

Analysis of Scanning Electron Microscopy

De-lipidized Hair :

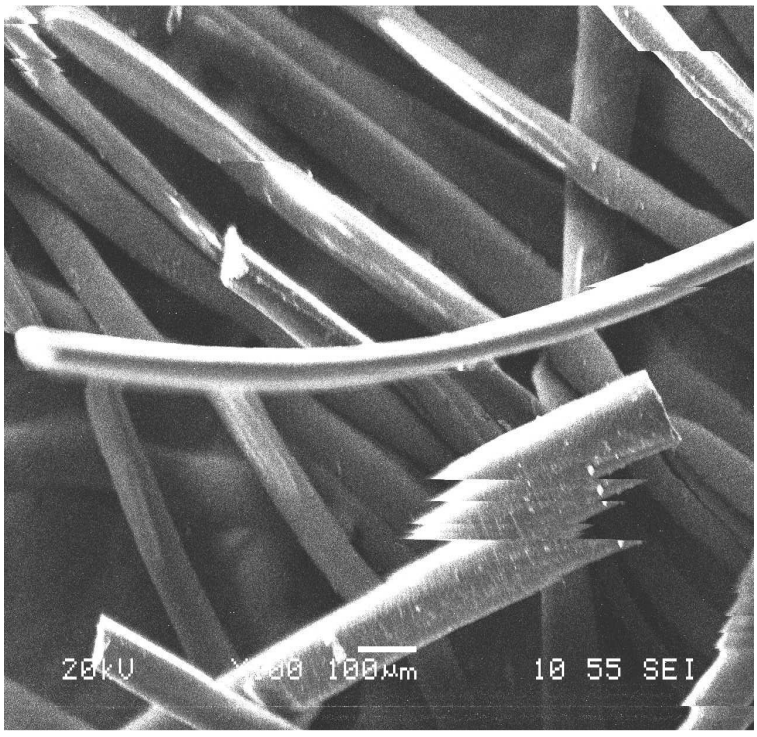

Figure 2 - SEM of Delipidized Hair

De-proteinized Hair :

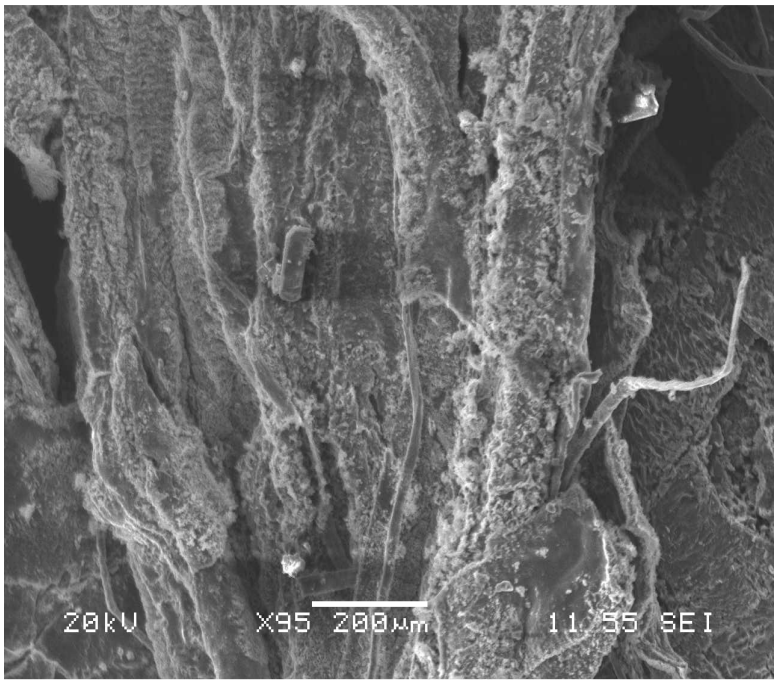

Figure 3 - SEM of Deproteinized Hair

Extracted Protein :

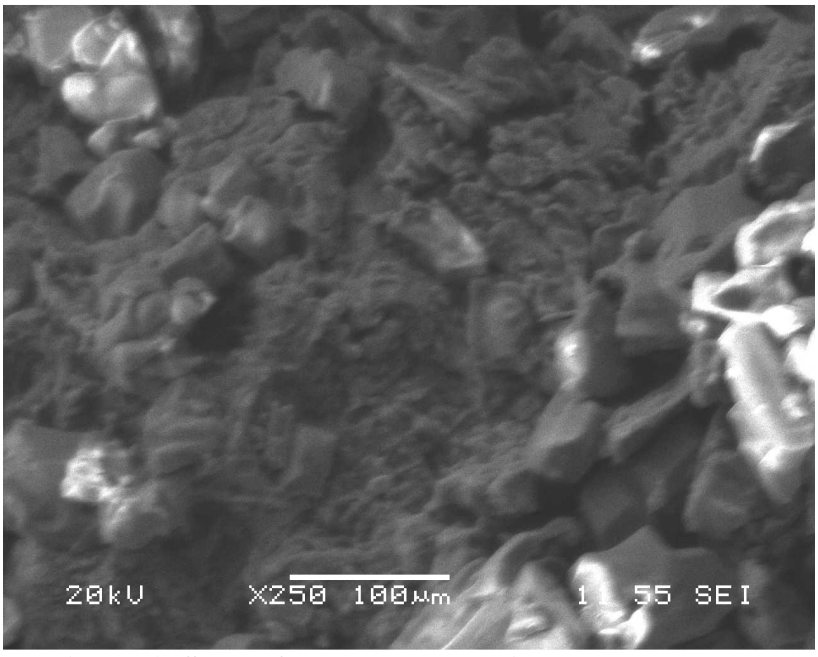

Figure 4- SEM of Extracted Protein

4.2 Fourier Transform Infrared Spectroscopy

Infrared spectroscopy is among the oldest and established experimental techniques which has been used for the characterisation and analysis of the secondary structure of polypeptides and proteins.[26-30]. Since all the frequencies are measured all together in one go, the majority of the measurements by FT-IR are done in few seconds. Better Sensitivity, mechanical simplicity, convenience involved and internal calibrations are some of the merits of FT-IR. It is a non destructive method which requires easy and less sample preparation. FTIR Spectroscopy is acknowledged as an important and efficient tool for the assessment of protein conformation in water-based solution. Moreover, in the past decade, it has greatly helped in the studies of protein secondary structure and protein dynamics [29−31]. The FT IR analysis can be done to acquire spectra of proteins in a wide range environment.

The polypeptide and protein repeat units provide nine chief IR absorption bands, i.e., amide A, B, and I−VII. The amide I and II bands are the two most prominent vibrational bands of the protein backbone [28−30]. The most responsive spectral area to the protein secondary structural components is the amide I band (1700−1600 cm−1), which is due almost entirely to the C=O stretch vibrations of the peptide linkages (approximately 80%). The frequencies of the amide I band components are found to be correlated closely to the each secondary structural element of the proteins. The amide band, in contrast, derives mainly from in-plane NH bending (40−60% of the potential energy) and from the CN stretching vibration (18−40%) [28], showing much less protein conformational sensitivity than its amide I counterpart [28]. Other amide vibrational bands are very complex depending on the details of the force field, the nature of side chains and hydrogen bonding, which therefore are of little practical use in the protein conformational studies.

Data Analysis

High sensitivity to small variations in molecular geometry and hydrogen bonding patterns makes the amide I band uniquely useful for the analysis of protein secondary structural composition and conformational changes [29, 32]. In the amide I region (1700−1600 cm−1), each type of secondary structure gives rise to a C=O (80 %) stretching frequency due to unique molecular geometry and hydrogen bonding pattern, with little in-plane NH bending (<20%) [28]. However, other than the distinctive absorbance maxima, the observed amide I bands of proteins are usually featureless, due to the extensive overlap of the broad underlying component bands, which lie in close proximity to one another and are instrumentally irresolvable. The overlap of secondary structural components is significant in the amide I region, even after mathematical resolution enhancement. Some investigators considered that the valley between two adjacent peaks of equal intensity must be 20% lower than the peak tops that could be resolved [26,33].

Table 2 - FTIR Band Assignment of Keratin (By researchers in previous Analysis)[34]

S.No	Absorption Peak (/cm)	Inference
1	1700−1600	Amide I band, C=O (80 %) and N-H bending (20%)
2	1654 -1658	α-helix
3	1688, 1680, 1672, and 1666	β-turn structures
4	1648±2	random coil conformation

Table 3- Corresponding Band Assignment for the Extracted Protein

S.No	Absorption Peak (/cm)	Inference
1	3420.26	vibratory absorption peak of imino group N-H In Keratin molecule
2	1650.10	stretching vibration absorption of amide I (C=O)
3	1547.73	stretching vibration absorption (characteristic absorption band of antipeptide bond structure) of amide II (mainly deformation vibration in NH-plane)
4	1395.45	absorption band of cis-peptide bond
5	1128.23	bending vibration peak of C-O-C bond
6	684.09	bending vibration peak of N-H bond

From the tables above, it can be concluded that the protein extracted from the Human Hair, using Shindai Method could be Keratin. The absorption peaks of the samples under analysis correspond to the FTIR standard peaks for Keratin Proteins. We have also predicted the bonds present in the samples at different peak values. The bonds are suggestive of the Keratin structure present in the sample analysed. Here, we have taken two samples, both being the

extracted protein. These samples were acquired during two different experiments to check the coherence in their peak values and hence structures. As seen from the FTIR analysis of the two samples shown below, it can be safely said that the two samples have similar structural arrangements.

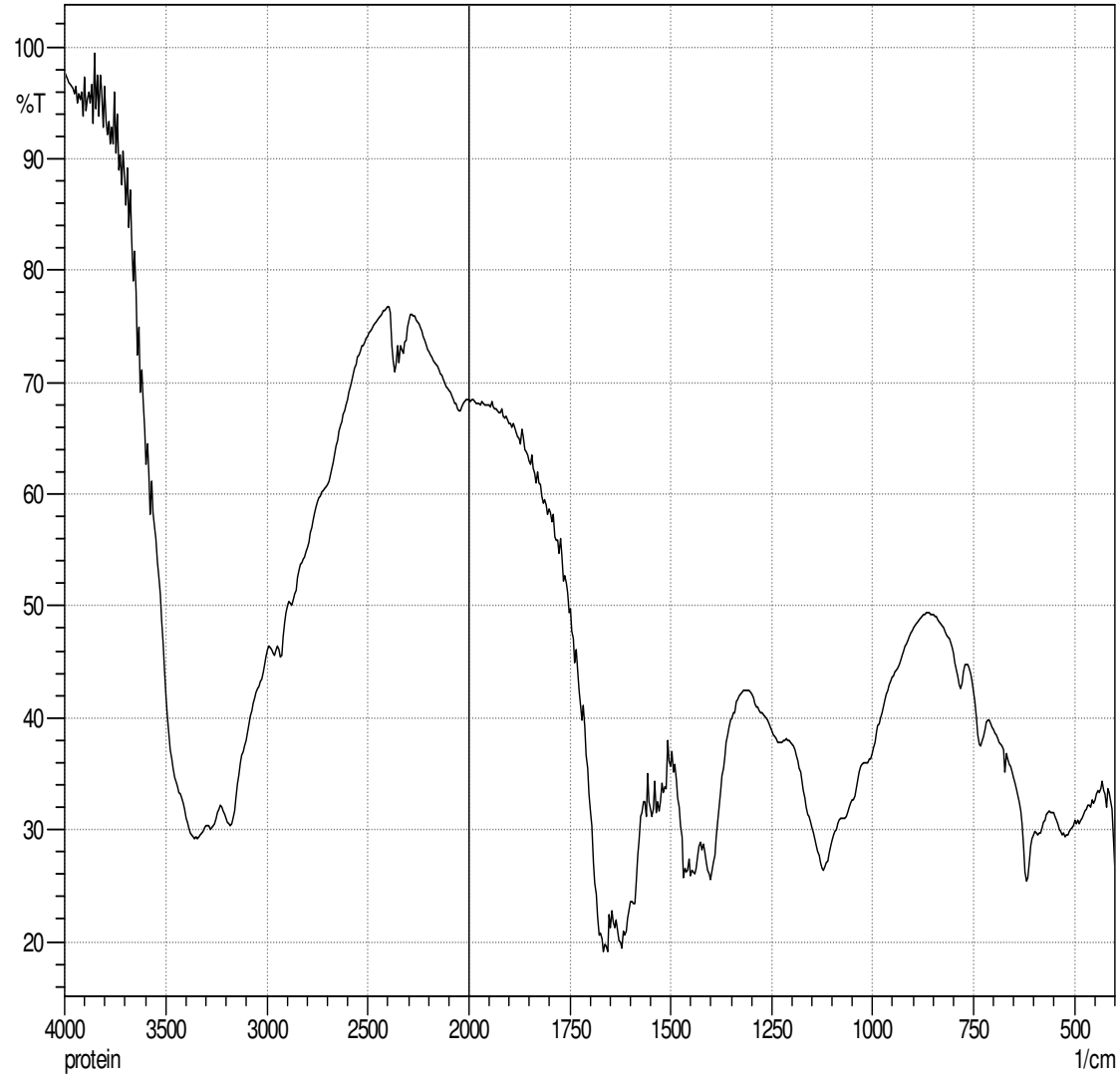

Figure 5 -FT-IR of Extracted Protein Sample 1

(X – Wave Number, Y – Percentage Transmittance)

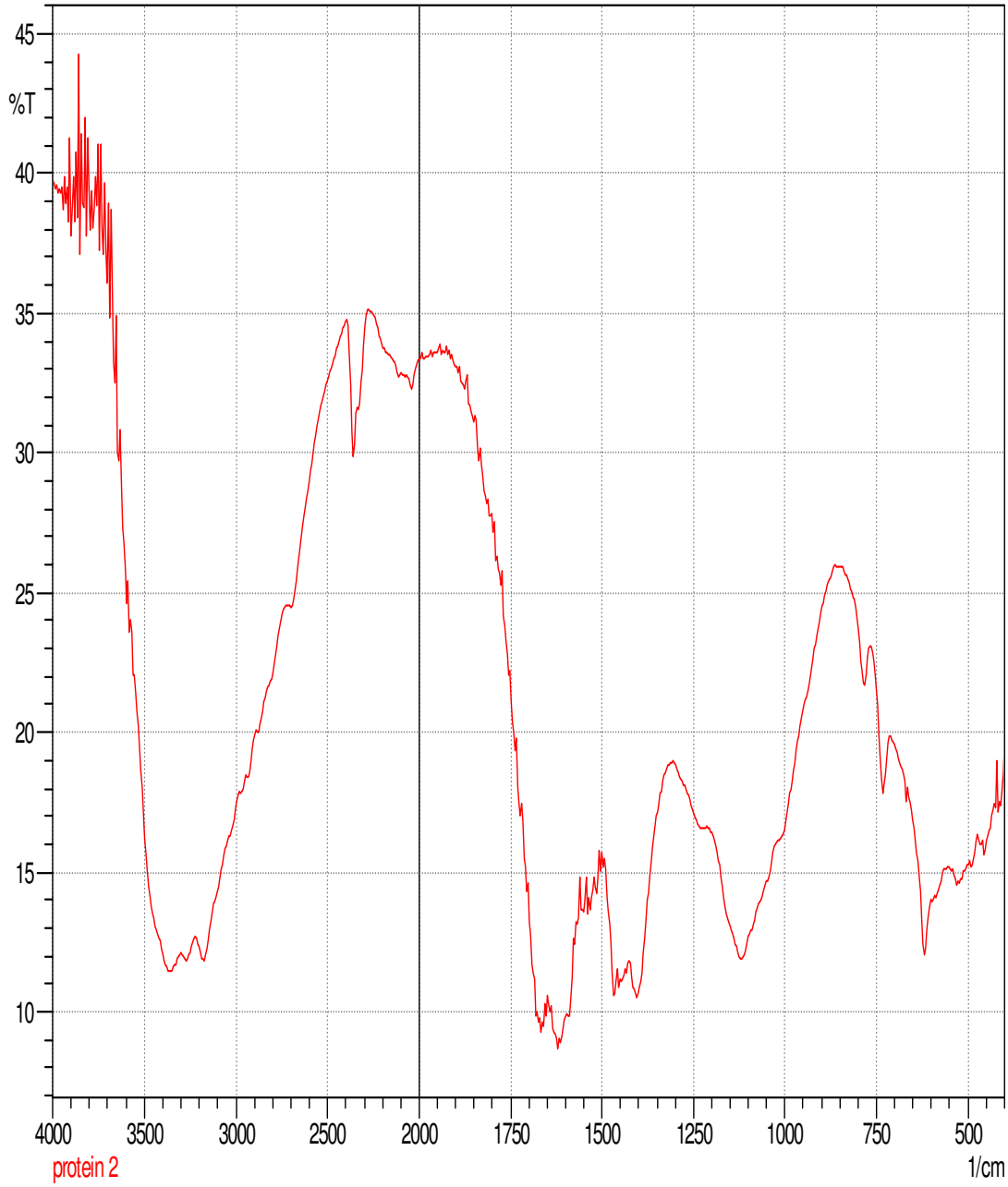

Figure 6- FT-IR of Extracted Protein Sample 2

(X – Wave Number, Y – Percentage Transmittance)

4.3 X-ray Diffraction:

X-ray Diffraction for protein like structures:

Is the alpha–beta transition of keratin -a transition of alpha-helices to beta-pleated sheets? In situ XRD studies [35]**.**

For the purpose of investigating the mechanism of the alpha–beta transition occurring in keratinous fibres, in -situ XRD experiments has been carried out. The outcome proved that the alpha to beta transition does not occur simultaneously upon stretching. This finding indicates the conventional interpretation that the a–b transition represents a helical molecular chain transferring into a b-pleated sheet may not be the case. In situ XRD experiments have been carried out while keratinous fibres are stretched and held. This finding indicates that conventional a-helix/b-pleated sheet configurational interpretation shall not be the case. An alternative interpretation is that a crystal transformation between different crystal modifications for the a–b transition appears to be more consistent to explain the observed experimental facts. Further investigations are considered necessary in order to entirely elucidate the mechanism of the– b transition.

Preparation and Characterization of *Bombyx mori* Silk Fibroin and Wool Keratin [36]

Wool and silk are eminent protein fibers. Their mechanical property, environmental stability, and biocompatibility render them significance. Based on the properties, they can be used in many biomedical applications. They can be used in the form of fibers, membranes or films. It is essential to differentiate these proteins in their solid and solution states in order to use them as biomaterials. Classification is necessary because morphological features and structural characteristics have immense influence on the mechanical and physical properties of these newly generated forms of proteins. Here wool keratin and silk fibroin were first dissolved. The solution's secondary structures and behaviour properties were compared and analyzed.

It was observed that keratin is more stable in solution state and in solid state it acts as amorphous. X-ray diffraction (XRD) was done in micro-diffraction mode with CuK radiation (_1.5418 Å) with geometry, a 2-mm-sized beam, and a two-dimensional detector (Bruker) with 1024 _ 1024 pixels. Frames of data were collected for 1 min each, integrated and merged to produce a diffraction pattern from 20° to 70°. The sample-to-detector distance was 1.3 m. The 2-D diffraction patterns were transformed into a 1-D radial average of the scattering intensity. The XRD results for the regenerated keratin film showed a broader peak, shifted 2_ degrees with lower intensity scores, indicating that the keratin film was more amorphous than the fibroin film. There were also other high-intensity regions for both protein structures: 28° for fibroin and 32° and 34°for keratin. Most probably this pointed to the different types of crystalline structures present.

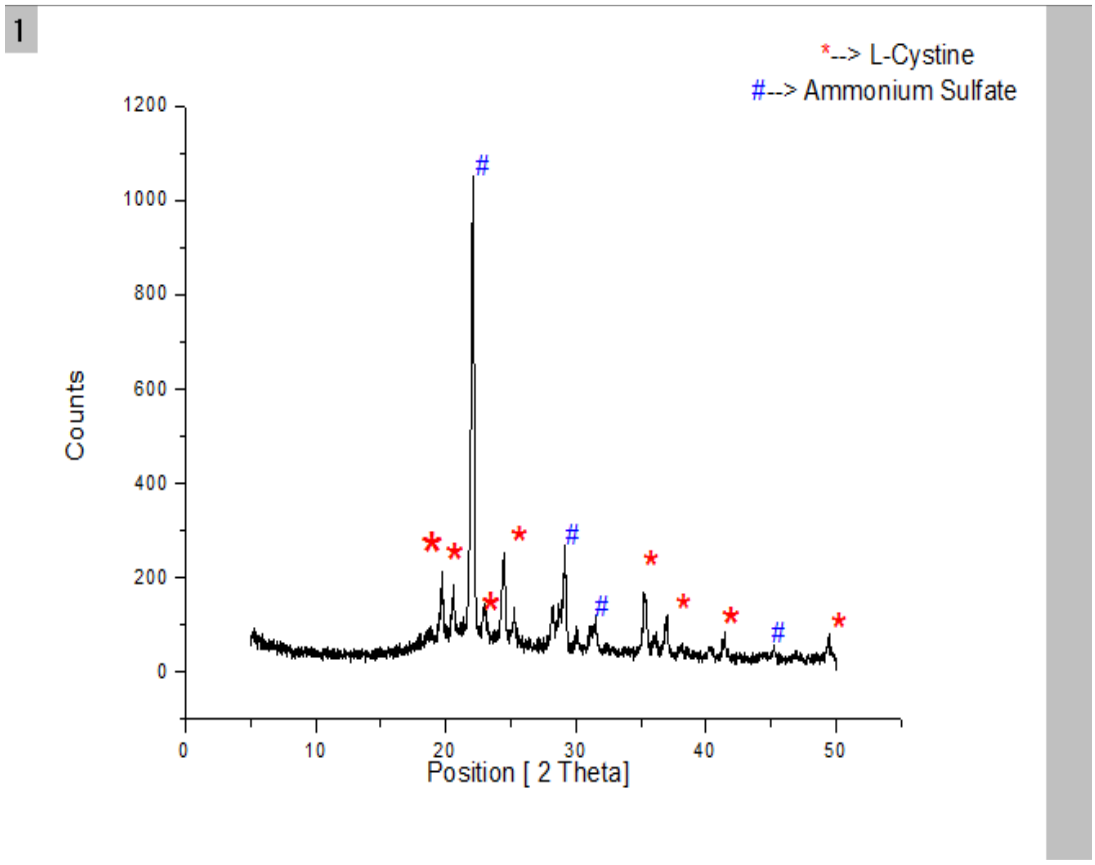

Figure 7 - XRD of Extracted Protein Sample

Table 4- Peak List

Pos. [°2Th.]	Height [cts]	FWHM [°2Th.]	d-spacing [Å]	Rel. Int. [%]
19.7178	90.54	0.2400	4.49882	13.39
20.5735	79.13	0.1440	4.31359	11.71
22.0941	675.92	0.2160	4.02004	100.00
22.9660	51.47	0.2880	3.86935	7.61
24.4272	124.90	0.2400	3.64109	18.48
25.2232	45.30	0.2880	3.52797	6.70
28.2357	56.49	0.2880	3.15804	8.36
29.1798	138.25	0.1200	3.05797	20.45
30.0599	20.64	0.2880	2.97041	3.05
31.4813	49.32	0.1440	2.83947	7.30
35.2924	79.88	0.2880	2.54108	11.82
36.1801	22.28	0.2880	2.48074	3.30
36.9234	46.93	0.3840	2.43249	6.94
40.2790	13.22	0.5760	2.23725	1.96
41.4381	30.83	0.3360	2.17730	4.56
45.2298	19.29	0.2880	2.00319	2.85
46.9260	12.18	0.2160	1.93466	1.80
49.4073	29.96	0.2880	1.84315	4.43

Table 5 - Pattern List

Visible	Ref. Code	Score	Compound Name	Displacement [°2Th.]	Scale Factor	Chemical Formula
*	23-1663	Unmatched Strong	L-Cystine	0.000	6.987	C6 H12 N2 O4 S2
*	26-0116	13	Arsenic	0.000	0.929	As

The peak list of the XRD obtained was studied with the reference of analysis done by various authors, particularly pertaining to Keratin protein. While analysing the plot above, we can say that the presence of L-cystine suggests that Keratin structure is present in the sample. The ammonium sulphate peak to be seen in the graph is because of the use of ammonium sulphate salting technique for protein extraction. The plot is suggestive of some structural derangement in the sample. Since the presence of coiling structure is confirmed by a peak seen at 5.4 Armstrong but the sample here shows a peak at 5.1 Armstrong, it can only be said that there is lack of coiling as well as secondary and tertiary structure. Crystallization of the sample might have given a better result.

4.4 Energy Dispersive X-ray spectroscopy Analysis

Arsenic Absorbed Hair

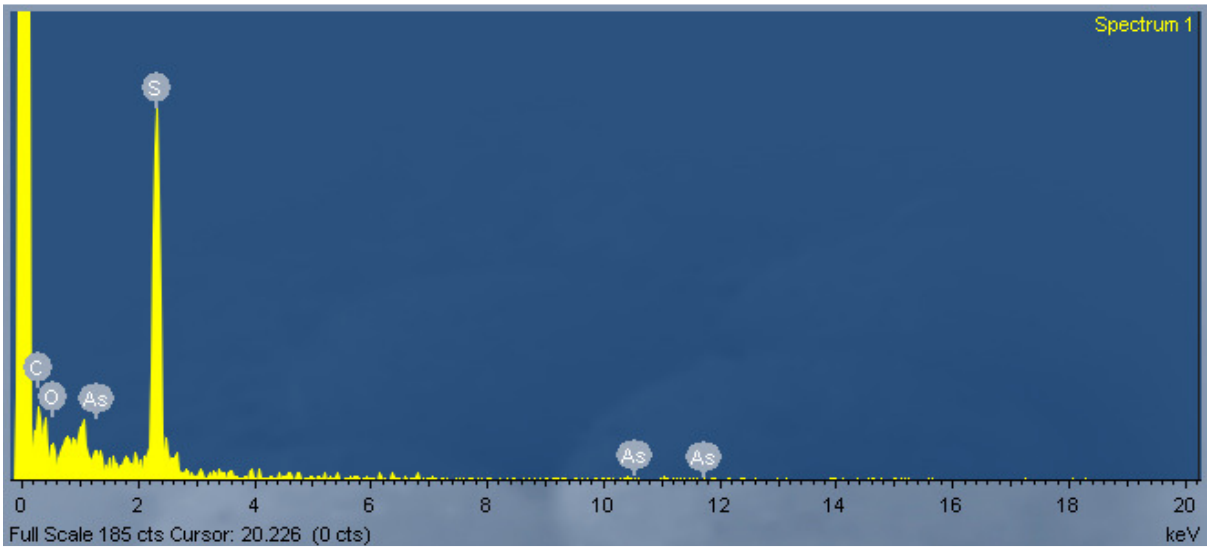

Figure 8 - Spectrum obtained by EDX Of Arsenic Absorbed Hair

The As absorption by de-proteinized hair is shown above. There is an increase in the absorption by the human hair.

Extracted Protein

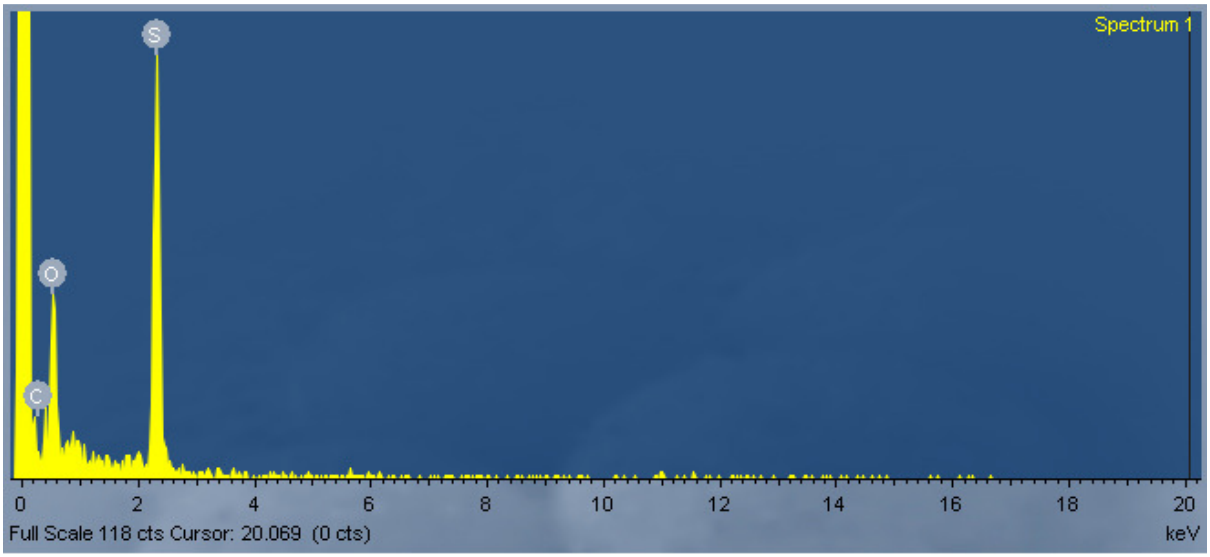

Figure 9- EDX analysis of Protein extracted from de-lipidized hair

The figure above shows that the protein obtained has huge amount of Sulphur content. Thus helping us assume that the keratin protein has been extracted from hair because of the presence of Cystine bonds.

4.5 Differential Scanning Calorimetry

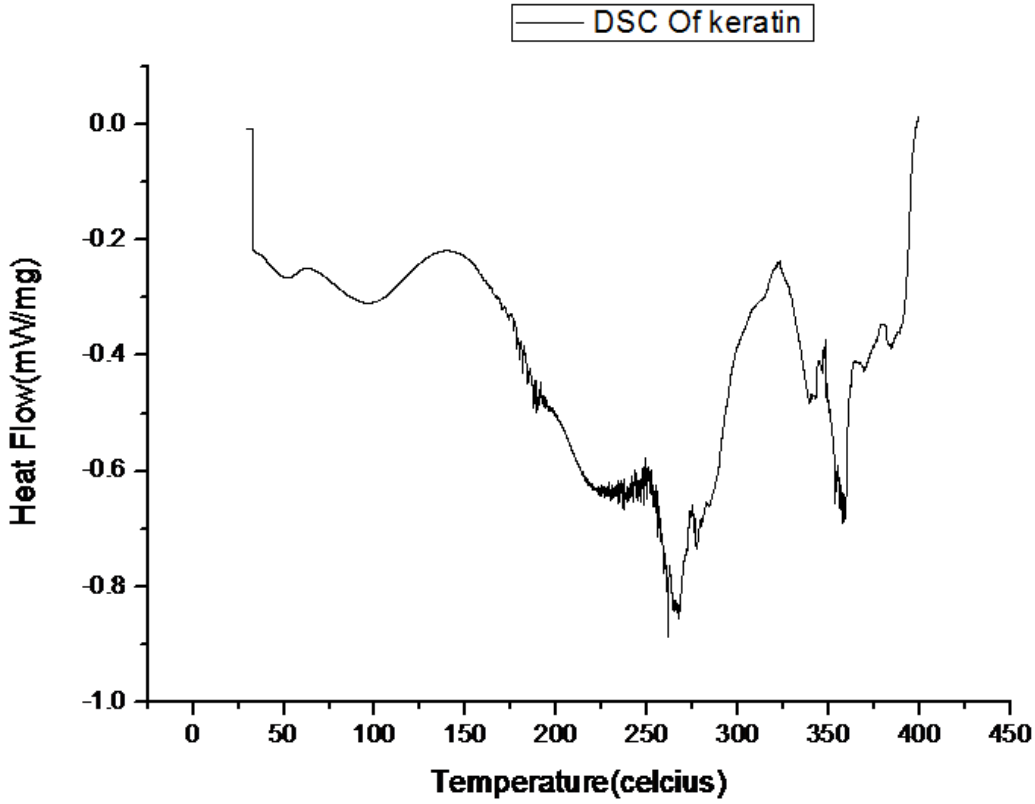

Figure 10 - DSC Of Extracted Protein

Application of differential scanning calorimetry in hair samples as a possible tool in Forensic Science [37]

Here they have used the Differential Scanning Calorimetry to study hair's thermal behaviour. This study is done to check the possibility of presence of a particular entity through the curves obtained from a data bank. The data bank was build up by the hair samples collected from the officials and students of Instituto de Química de Araraquara, UNESP.

The thermal analytical systems, Differential Scanning Calorimetry (DSC) and Differential Thermal Analysis (DTA) were used with success in the description and characterization of many products such as mammoth's ivory or elephant's ivory [38]; soils [39];wool [40], etc. The aim of the study is the verification of the potentiality of the DSC techniques to identify an individual through his/ her hair DSC curves with the aid of a bank data.

The isotherm observed above 300ºC, which suggests several overlapping events, is attributed to the hair's organic matter oxidation. In spite of the great similarity in the profile of these curves slight differences, are observed above 300ºC.The DSC curves of electrospun keratin/PEO samples was compared with the films produced by casting using the same solution. This was done to highlight the structural changes which were induced by the electrospinning process. It was found that at 60°C, the endothermic peaks overlapped. The reason behind the overlapping was the fusion of PEO crystalline phase and the evaporation of water, absorbed by keratin. It has been observed while comparing the water evaporation temperature of the keratin/PEO film and the keratin/PEO nanofibres, that evaporation in the keration/PEO occurs at lower temperature (50°C in the nanofibres and 80°C in the film). This could be due to Keratin-based Nanofibres 149 to the high surface/volume ratio of the nanofibres which promotes water evaporation even at lower temperatures. The DSC analysis the electrospun PEO exhibits a slight increase of the melting point. It is believed that the high stretching due to the electrospinning process promotes the orientation of the long polymer chains of PEO. This high degree of order shifts the melting point to a higher temperature. The endothermic events observed in the range of 200-350°C are attributed to protein denaturation followed by protein degradation (Spei & Holzem, 1990).

Table 6- Characterization of Peaks observed [41, 42, 43]

S.No	Peak Temperature (°C)	Peak	Inference
1	200–250	endothermal peak	α-helix denaturation, area under the curve is α-helix content
2	278	fall of endothermal peak	denaturation of β structures
3	223	fall of denaturation peak	well developed crystalline structure

On the basis of the analysis done earlier it was concluded by the authors that because of the high molecular orientation in the nano fibers, the alpha crystalline structure was not well developed. The DSC of the nanofiber showed different peak temperatures, when compared with the protein film [44, 45].The extracted protein is analysed using the Differential Scanning Calorimetry. A plot is made between the Temperature and the Heat flow. Temperature ranging from room temperature to 400° C and the following inferences were made:

Denaturation peak, which depicts the amount of H bound water present in Keratin, was found at 100° C. The peak/ dip at 250 °C represent the crystallinity or melteing temperature of keratin. Many peaks and dips can be seen at temperatures greater than 300 °C, which might be due to charring. Considering the similarities present between the DSC analysis of our sample and that of Keratin protein, it was concluded that the protein extracted was Keratin.

4.6 Arsenic Absorption Testing

At first a solution of arsenic trioxide is run through a sample of normal hair. The test strip is dipped as per the indications given with the QUANTOFIX ARSEN 10 KIT. This is taken as control. It is the maximum concentration that the Test can detect. After this the arsenic trioxide solution is run through the mass of protein extracted from human hair. It was observed that the extracted protein absorbed arsenic. Hence proving that the protein extracted from human hair is arsenic absorbent. The control was found to be absorbing 0.5 ppm whereas the sample absorbed 0.05 ppb of As.

Figure 11 - Extracted Protein

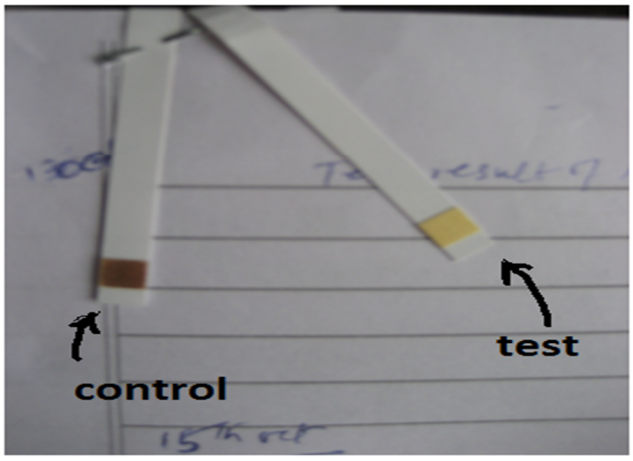

Figure 12 - Testing Strips

CONCLUSION

The absorption of Arsenic by human hair protein has been analysed. The analysis suggests that Keratin Protein has considerable ability to absorb arsenic from water. Protein extraction from human hair was carried out. Various studies have been done on the extracted sample to characterise it. These tests have shown positive results, indicating that the protein extracted from the hair is Keratin. The data obtained in these tests showed no or very little variation from that of Keratin. These tests such as FTIR, DSC, XRD, DSC etc have helped in the understanding of the structural and chemical composition of the sample obtained. This work may further be extended to obtain more data for the design of a water purifying system made up of extracted Keratin protein from human hair. Intensive analysis is required for creating such a device which should be efficient, reusable and at the same time, low in cost. Such an invention would save the lives of many, who are dreadfully suffering from the diseases caused by Arsenic in their drinking water supply system and hence, would serve the mankind.

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36. Preparation and Characterization of Bombyx mori Silk

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